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REGULAR ARTICLE

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Signal transduction and regulation of melatonin synthesis in bovine pinealocytes: impact of adrenergic, peptidergic and cholinergic stimuli

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Abstract Limited studies of the regulation of pineal melatonin biosynthesis in ungulates indicate that it differs considerably from that in rodents. Here we have investigated several signal transduction cascades and their impact on melatonin synthesis in bovine pinealocytes. Norepinephrine increased the intracellular calcium ion concentration ($[Ca^{2+}]_i$) via α_1-adrenergic receptors. Activation of β-adrenergic receptors enhanced cAMP accumulation and rapidly elevated arylalkylamine *N*-acetyltransferase (AANAT) activity and melatonin secretion. The β-adrenergically evoked increases in AANAT activity were potentiated by α_1-adrenergic stimulation, but this was not seen with cAMP or melatonin production. PACAP treatment caused small increases in cAMP, AANAT activity and melatonin biosynthesis, apparently in a subpopulation of cells. VIP and glutamate did not influence any of these parameters. Activation of nicotinic and muscarinic acetylcholine receptors increased $[Ca^{2+}]_i$, but did not alter cAMP levels, AANAT activity or melatonin production. Our study reveals that discrete differences in pineal signal transduction exist between the cow and rodent, and emphasizes the potential importance that the analysis of ungulate pinealocytes may play in understanding regulation of pineal melatonin biosynthesis in primates and man, whose melatonin-generating system appears to be more similar to that in ungulates than to that in rodents.

Keywords Acetylcholine · Arylalkylamine *N*-acetyltransferase · Biological rhythms · Norepinephrine · PACAP · Bovine

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Introduction

In all vertebrates, melatonin is rhythmically produced in the pineal gland, with high levels occurring at night. Accordingly, melatonin can be considered as the hormonal message for darkness. Although this pattern is highly conserved among vertebrates, the signal transduction cascades and the molecular mechanisms that regulate melatonin production differ strikingly. These differences are particularly obvious when mammals, birds, and fish are compared (Klein et al. 1997; Korf et al. 1998).

Mammalian pineal signal transduction cascades have been investigated most intensely in the rat (Klein 1985; Klein et al. 1997; cf. Korf et al. 1996, 1998). Norepinephrine (NE) is the key physiological regulator of melatonin synthesis, but other neuroactive substances have been shown to influence the melatonin-generating system. These include vasoactive intestinal peptide (VIP) (Kaneko et al. 1980; Yuwiler 1983; Simonneaux et al. 1990; Schomerus et al. 1996), pituitary adenylate cyclase-activating polypeptide (PACAP) (Simonneaux et al. 1993; Chik and Ho 1995; Olcese et al. 1996; Schomerus et al. 1996), as well as acetylcholine (ACh) (Schomerus et al. 1995; Letz et al. 1997; Yamada et al. 1998a).

NE is released in the rat pineal gland from sympathetic nerve endings at the onset of darkness (Drijfhout et al. 1996); it activates pinealocytes through α_1- and β-adrenergic receptors and subsequent increases in the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) (Sugden et al. 1987; Schaad et al. 1993; Schomerus et al. 1995) and cyclic AMP (Vanecek et al. 1985). Increases in cAMP concentration are essential for activation of melatonin production. Elevated cAMP levels result in protein ki-

nase A-dependent phosphorylation of the stimulatory transcription factor cyclic AMP response element (CRE)-binding protein (CREB) (Roseboom and Klein 1995; Tamotsu et al. 1995; Maronde et al. 1999). Phosphorylated (p) CREB binds to a CRE element in the arylalkylamine *N*-acetyltransferase (AANAT) gene (Baler et al. 1997), resulting in increased transcription and accumulation of AANAT mRNA (Roseboom et al. 1996). AANAT is of special importance because changes in the activity of this enzyme control the daily rhythm in melatonin synthesis in vertebrates (Klein and Weller 1970). The daily fluctuations in AANAT mRNA are associated with similar changes in AANAT protein levels and AANAT activity (Klein 1985; Klein et al. 1997). In addition to this transcriptional mechanism, which controls AANAT protein and activity in the rat, there is posttranslational regulation of AANAT protein levels via cAMP-dependent inhibition of proteasomal proteolysis in all vertebrates investigated so far (Gastel et al. 1998; Schomerus et al. 2000; Falcón et al. 2001; Iuvone et al. 2002); this mechanism involves phosphorylation-dependent binding of AANAT to 14-3-3 proteins, which shields AANAT from proteolysis (Ganguly et al. 2001; Obsil et al. 2001).

Marked differences in the mechanism involved in regulating AANAT activity are seen when rodents are compared to ungulates. The most obvious is the difference in the night/day levels of AANAT mRNA: in the rat, the night/day ratio is >100 (Roseboom et al. 1996), whereas it is ~1.5 in ungulates (Coon et al. 1995). In the latter, AANAT protein levels are regulated primarily at the posttranslational level by controlled proteasomal proteolysis (Schomerus et al. 2000). Similar mechanisms may also operate in primates because the AANAT mRNA night/day ratio in the Rhesus monkey is small (Klein et al. 1997) and the dynamics of melatonin secretion are similar to those in ungulates (Arendt 1995).

This distinct difference between rodents and ungulates provides clear evidence that models of pineal signal transduction based solely on the rat may not apply to mammalian species other than rodents. To clarify how melatonin production might be regulated in the human pineal gland, it is important to obtain a full understanding of pineal signal transduction cascades in other model systems. The experiments presented in this report were designed to provide an understanding of the influence of various stimuli (adrenergic, cholinergic, peptidergic, glutamatergic) on $[Ca^{2+}]_i$, cAMP accumulation, AANAT activation, and melatonin release in another model system, the bovine pinealocyte (Schomerus et al. 2000). Our results offer new insights into vertebrate pineal signal transduction.

Materials and methods

Materials

Drugs and chemicals were obtained from the following sources: norepinephrine, isoproterenol, phenylephrine, acetylcholine, nicotine, atropine, *d*-tubocurarine, nifedipine, carbachol, glutamate, 3-isobutyl-1-methylxanthine, tryptamine hydrochloride, poly-L-lysine (Sigma, Deisenhofen, Germany); forskolin, VIP, PACAP (Calbiochem, Bad Soden, Germany); Bay K 8643, oxotremorine-M (Biotrend, Köln, Germany); papain (Boehringer, Mannheim, Germany); bisubstrate inhibitor (BSI, coenzyme A-*S*-acetyltryptamine; RBI, Natick, Mass., USA); Fura-2/AM (Molecular Probes, Leiden, Netherlands); Rp-8-CPT-cAMPS (Biolog, Bremen, Germany).

Cell culture

Bovine pineal glands were obtained from male and female animals at an abattoir in the vicinity of Frankfurt/Main, Germany. The glands were dissected between 0600 h and 1000 h within 15 min after death and brought to the laboratory on wet ice. Dissociated pinealocytes were prepared as described (Schomerus et al. 2000) and plated into 96-well culture dishes (Nunc, Wiesbaden, Germany; ~200,000 cells per well) or onto poly-L-lysine-coated coverslips. The viability of the adherent cells, assessed by trypan blue exclusion, was found to be ~95%. Immobilized pinealocytes were incubated in DMEM/F12 growth medium containing fetal calf serum (10%), HEPES (10 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), ascorbic acid (100 µg/ml), and amphotericin B (1 µg/ml) under 92% air/8% CO_2 at 37 °C. After five days in culture, the cells were incubated in serum-free growth medium for 1 h prior to the onset of the experiments. All treatments were done in serum-free growth medium. The experiments were performed throughout the year; there was no evidence for a seasonal variation in the responses studied.

$[Ca^{2+}]_i$ measurements

Pinealocytes immobilized on coverslips were loaded with fura-2/AM (2.5 µM) for 15 min at 37 °C in growth medium containing 10% fetal calf serum. Subsequently, coverslips were rinsed with saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4), placed into a superfusion chamber mounted on the heatable stage of an Axiovert 100 microscope (Zeiss, Germany), and superfused with saline at 37 °C. Data acquisition and analysis of fluorescence signals from single cells were performed by use of an Attofluor calcium imaging system (Zeiss, Germany; see Schomerus et al. 1995). For stimulation of the cells under Ca^{2+}-free conditions, $CaCl_2$ was omitted from the saline and EGTA (5×10^{-4} M) was added. In some experiments, the semiquantitative ratio values were converted to approximate calcium concentrations by performing in vitro calibrations subsequent to the experiments. At least 300 cells were analysed in each experiment.

Cyclic AMP measurements

Cyclic AMP was measured in cell extracts and in mixed preparations of media and cells. For measurements of intracellular cAMP, cells were treated with the indicated drugs for 15 min. Thereafter, the supernatants were aspirated and ice-cold 80% ethanol was added to the cell pellets for 30 min. To remove precipitates, the samples were centrifuged for 10 min (10,000 *g*), and the supernatants were taken to dryness under vacuum. Cyclic AMP was acetylated and its total amount determined by use of a commercial ELISA (IHF GmbH, Hamburg, Germany). For measurements of total accumulated cAMP, cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 5×10^{-4} M; 15 min) and then stimulated with the indicated drugs for 15 min in the presence of IBMX. Cells and the corresponding media were collected together, and ethanol was added to a final concentration of 80%. The samples were kept on ice for 30 min, and cell debris were removed by centrifugation for 10 min (10,000 *g*). Cyclic AMP was determined as described above. The cAMP measurements were verified in control experiments. The cAMP values ob-

tained remained basically unchanged, irrespective of whether cAMP was extracted with 80% ethanol or with 6% trichloric acid followed by removal of trichloric acid with diethyl ether. Furthermore, exogenous cAMP added to the samples was recovered with an average rate of ~90%, indicating that there was no substantial loss of cAMP during the extraction procedure (data not shown).

AANAT immunocytochemistry

For immunocytochemical demonstration of AANAT, cells were treated with the indicated drugs for 6 h. Untreated preparations served as controls. Cells were fixed with 4% glutaraldehyde (10 min) and incubated with a highly specific polyclonal antibody raised against a synthetic peptide corresponding to ovine $AANAT_{1–25}$ (serum 3343; 1:25,000), which recognizes bovine AANAT (Schomerus et al. 2000). Binding of the primary antibody was visualized using the ABC method, with a biotin-conjugated anti-rabbit IgG (Sigma, Deisenhofen, Germany) as the second antibody, a horseradish peroxidase-conjugated streptavidin complex (Sigma, Deisenhofen, Germany), and diaminobenzidine as the chromogen. Antiserum specificity was validated by preabsorption of the antiserum (overnight, at room temperature) with 100-fold molar excess of the antigenic peptide. The resulting antiserum did not generate an immunopositive signal with preparations of NE-treated cells.

Immunoblot analysis of AANAT

Cells were treated for 6 h with the indicated drugs, removed from 96-well culture dishes, collected by centrifugation (30 s, 10,000 *g*), and frozen on solid CO_2. Sample preparation, immunoblotting and immunodetection were performed using the AANAT antiserum 3343 (1:25,000) as described (Schomerus et al. 2000). Analysis of the protein concentration in the cell extracts (Bradford 1976) indicated that equal amounts of protein were loaded in each lane.

AANAT activity assay

AANAT activity was measured in homogenates from cells treated for 6 h with the indicated drugs. For preparation of cell homogenates, cells were collected from 96-well culture dishes and pelleted by centrifugation (30 s, 10,000 *g*). The supernatants were removed, and the cell pellets were frozen on solid CO_2. After thawing, the broken cells were incubated at 37 °C in 0.1 M ammonium acetate, pH 6.8, containing tryptamine (0.1 mM) and [1-^{14}C]acetyl coenzyme A (10^{-3} M; specific activity 55 µCi/µmol; New England Nuclear, Köln, Germany). After 20 min, the reaction was stopped by adding 1 ml chloroform. The organic phase containing radiolabelled *N*-[^{14}C]acetyltryptamine was washed twice with 0.2 ml of chloroform-saturated 0.1 M ammonium acetate, pH 6.8. Finally, a 0.5 ml sample of the chloroform phase was taken to dryness, and radioactivity was determined.

Radiolabelled reaction products were verified by thin-layer chromatography using authentic *N*-acetyltryptamine as a marker. In both controls and stimulated cells, synthesis of *N*-[^{14}C]acetyltryptamine was totally blocked by an AANAT-specific bisubstrate inhibitor (BSI, coenzyme A-*S*-acetyltryptamine; 10^{-4} M; Khalil and Cole 1998). Activity values were normalized against the amount of total protein determined using the method of Bradford (1976).

Melatonin secretion

Cells were treated with drugs for different time intervals as indicated. Thereafter, melatonin secreted into the supernatant was determined by a commercial ELISA (IHF GmbH, Hamburg, Germany) using biotinylated melatonin as a tracer. The detection limit of this assay is 2 pg melatonin/ml. For data presentation, the melatonin concentration in the supernatant of cells treated with NE (10^{-7} M) was set as 100%, and all other values were expressed as % of the value obtained after treatment with NE, unless otherwise indicated. The average melatonin concentration in the supernatants of the pinealocyte preparations after treatment with NE (10^{-7} M) ranged between 15 and 30 ng/ml.

Statistics

To minimize effects of inter-individual variation, pineal glands from at least three animals were pooled for a single cell preparation, and all data presented are based on at least three different preparations. Data from three individual experiments were statistically analyzed using an ANOVA, with subsequent Bonferroni tests, with $P<0.05$ as the criterion of significance and expressed as mean ± SEM.

Results

$[Ca^{2+}]_i$ measurements

NE treatment increased $[Ca^{2+}]_i$ in 75% of the pinealocytes (Fig. 1A–F). The lowest NE concentration found to increase $[Ca^{2+}]_i$ was 10^{-7} M. The most robust responses were obtained at 10^{-6} M NE (Fig. 1A). Calibration of the semiquantitative ratio values showed that $[Ca^{2+}]_i$ rose from basal levels of 75±10 nM to peak values of 440±60 nM (data not shown). The NE-induced $[Ca^{2+}]_i$ response was biphasic: immediately after the onset of the stimulus, $[Ca^{2+}]_i$ increased to a transient maximum; thereafter, $[Ca^{2+}]_i$ decreased, but remained significantly elevated above basal levels as long as the cells were exposed to the stimulus. When cells were kept in Ca^{2+}-free saline, NE treatment induced a short-lasting rise in $[Ca^{2+}]_i$, the amplitude of which was smaller than that observed in cells kept in Ca^{2+}-containing saline (Fig. 1B). NE treatment did not induce a $[Ca^{2+}]_i$ response in cells that were kept in Ca^{2+}-free saline and pretreated with 2 µM thapsigargin (data not shown). These results indicate that both extracellular and intracellular calcium pools contribute to the NE-evoked $[Ca^{2+}]_i$ response.

To identify the type of adrenoreceptor involved in the $[Ca^{2+}]_i$ response, cells were treated with α_1- (phenylephrine; PE) or β- (isoproterenol; ISO) specific adrenergic agonists. Like NE, PE treatment at a concentration of $\geq 10^{-7}$ M elicited a biphasic $[Ca^{2+}]_i$ response in ~70% of the cells, whereas ISO (10^{-6} M) treatment did not alter $[Ca^{2+}]_i$ (Fig. 1C). To investigate whether functional voltage-gated calcium channels are present in bovine pinealocytes, cells were treated with KCl (6×10^{-2} M; Fig. 1D) or Bay K 8643 (10^{-6} M; data not shown). In both cases, ~70% of the cells responded to the stimulus. There was no apparent $[Ca^{2+}]_i$ response of pinealocytes to VIP, PACAP (10^{-7} M each; Fig. 1D), or glutamate (10^{-3} M; data not shown).

In nearly all NE-responsive cells, ACh (10^{-4} M; Fig. 1E) or carbachol (10^{-4} M, data not shown) elicited an increase in $[Ca^{2+}]_i$. Treatment with cholinergic agents increased $[Ca^{2+}]_i$ about fivefold from basal levels (70±10 nM versus 370±50 nM), according to calibration of the semiquantitative ratio values. The majority of these cells also responded to both the nicotinic receptor agonist nico-

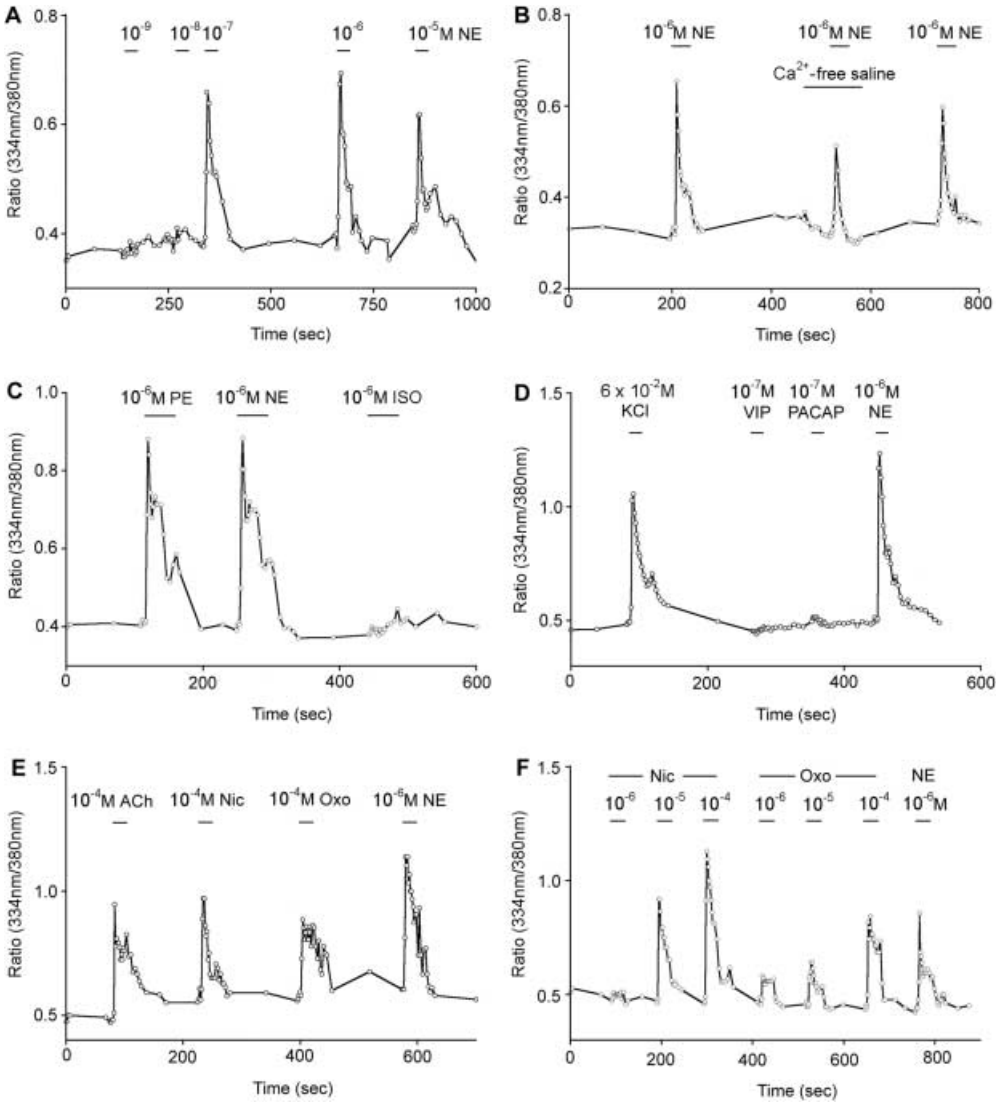

Fig. 1A–F $[Ca^{2+}]_i$ recordings from isolated bovine pinealocytes. **A** Norepinephrine (NE) increased $[Ca^{2+}]_i$ in a dose-dependent manner. The response was biphasic: after a transient maximum, $[Ca^{2+}]_i$ dropped to an elevated level persisting as long as the cells were exposed to NE. **B** When NE was applied in Ca^{2+}-free saline, the amplitude of the response was reduced, and $[Ca^{2+}]_i$ rapidly dropped to basal levels before NE was removed. **C** Phenylephrine (PE; 10^{-6} M), but not isoproterenol (ISO; 10^{-6} M) mimicked the $[Ca^{2+}]_i$ response induced by NE (10^{-6} M). **D** KCl (6×10^{-2} M) elicited a rise in $[Ca^{2+}]_i$ in most NE-responsive cells, whereas VIP (10^{-7} M) or PACAP (10^{-7} M) were ineffective. **E** The cholinergic agonists acetylcholine (*ACh*), nicotine (*Nic*), and oxotremorine-M (*Oxo*) (10^{-4} M each) elicited marked increases in $[Ca^{2+}]_i$ in cells that were also responsive to NE (10^{-6} M). **F** The $[Ca^{2+}]_i$ responses induced by nicotine (*Nic*) or oxotremorine-M (*Oxo*) were dose-dependent. All experiments were performed at least in triplicate with cells from different cell preparations. For further details, see Materials and methods

tine and the muscarinic receptor agonist oxotremorine-M (Fig. 1E). The threshold concentrations of nicotine and oxotremorine-M required to induce $[Ca^{2+}]_i$ responses were 10^{-5} M and 10^{-6} M, respectively (Fig. 1F). The response to 10^{-4} M nicotine could be blocked by the nicotinic receptor antagonist *d*-tubocurarine (10^{-4} M), by inhibitors of voltage-gated L-type calcium channels (nifedipine or verapamil; 10^{-4} M each) or application of nicotine in Ca^{2+}-free saline. The response to oxotremorine-M (10^{-5} M) could be abolished by the muscarinic receptor antagonist atropine (10^{-8} M; data not shown).

Accumulation of cAMP

Treatment with NE (10^{-7} M) increased the intracellular cAMP level about twofold within 5 min; cAMP remained elevated during the 6 h experimental period (Fig. 2). An increase in cAMP with a similar time-course was also induced by ISO (10^{-7} M), whereas PE (10^{-6} M) had no effect (Fig. 2). Our data extend previous results (Olcese et al. 1991) and indicate that β-adrenergic receptors mediate a long-lasting rise in intracellular cAMP in response to NE treatment. Treatment with the cAMP protagonist forskolin (10^{-5}–10^{-7} M) also increased intracellular cAMP levels in a dose-dependent manner (Fig. 3A).

In the following experiments, unless otherwise indicated, total cAMP accumulation (i.e. intracellular cAMP plus cAMP secreted into the medium) was measured in cell preparations treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 5×10^{-4} M). Addition of IBMX markedly increased the amplitudes of the cAMP responses. Treatment with 10^{-7} M NE or 10^{-7} M ISO for 15 min resulted in an eight- to 15-fold rise in cAMP accumulation over control values, whereas PE at concentrations of up to 10^{-6} M had no effect (Fig. 4A). This is consistent with the interpretation that phosphodiesterases continually destroy cAMP.

To investigate whether the β-adrenergically evoked rise in cAMP accumulation is potentiated by α_1-adrenergic stimulation, cells were treated with combinations of

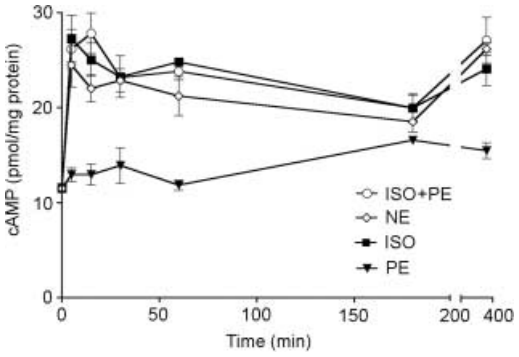

Fig. 2 Time-course study of intracellular cAMP levels after stimulation with adrenergic receptor agonists. Cells were stimulated with norepinephrine (*NE*; 10^{-7} M), isoproterenol (*ISO*; 10^{-7} M), phenylephrine (*PE*; 10^{-6} M), or a combination of *ISO* (10^{-7} M) + *PE* (10^{-6} M) without addition of IBMX. Data were determined in duplicate in each experiment; for data presentation, results from two independent experiments were pooled. For further details, see Materials and methods

ISO and PE. PE was given at a concentration of 10^{-6} M, a concentration shown to induce a strong $[Ca^{2+}]_i$ response (Fig. 1C) and a weak increase in melatonin secretion (Figs. 4D and 5C). Under these conditions, PE did not potentiate the rise in cAMP elicited by a range of ISO concentrations alone (Fig. 5A). Also in the absence of IBMX, a potentiating effect of PE (10^{-6} M) on cAMP accumulation induced by various concentrations of ISO ranging from 10^{-6} M to 10^{-10} M was not detectable (see Fig. 2, for a representative experiment with 10^{-7} M ISO).

Of the other drugs tested, only PACAP (10^{-7} M) caused a significant increase in cAMP (Fig. 4A). However, the efficacy of PACAP to elevate cAMP was distinctly lower than that of NE. VIP (10^{-7} M), Bay K 8643 (10^{-6} M), KCl (6×10^{-2} M) (Fig. 4A), glutamate (10^{-3} M), as well as the cholinergic agents ACh and carbachol (10^{-4} M each; data not shown) did not significantly alter cAMP.

Determination of AANAT protein levels and activity

In confirmation of our previous study (Schomerus et al. 2000), AANAT protein was readily detected in unstimulated bovine pinealocytes, it was found as a single 24-kDa signal in immunoblots (Fig. 4B), and the intensity of this signal was markedly increased in cells treated with NE (10^{-7} M). Moreover, we found that ISO treatment (10^{-7} M) for 6 h elicited a response very similar to that induced by NE, whereas PE (10^{-6} M) treatment was without effect (Fig. 4B).

As observed with AANAT protein levels, AANAT activity was strongly enhanced by treatment with NE (Figs. 3B, 4C, 6A and 7A; see Schomerus et al. 2000) or ISO, but not by PE (Fig. 4C). Forskolin (10^{-5} M) mimicked the effect of NE; however, lower concentrations of forskolin (10^{-6} M; 10^{-7} M) were distinctly less potent (Fig. 3B). This is noteworthy, because treatment with these concentrations of forskolin (10^{-6} M; 10^{-7} M) elevated cAMP to an extent similar to that induced by 10^{-7} M NE (see above).

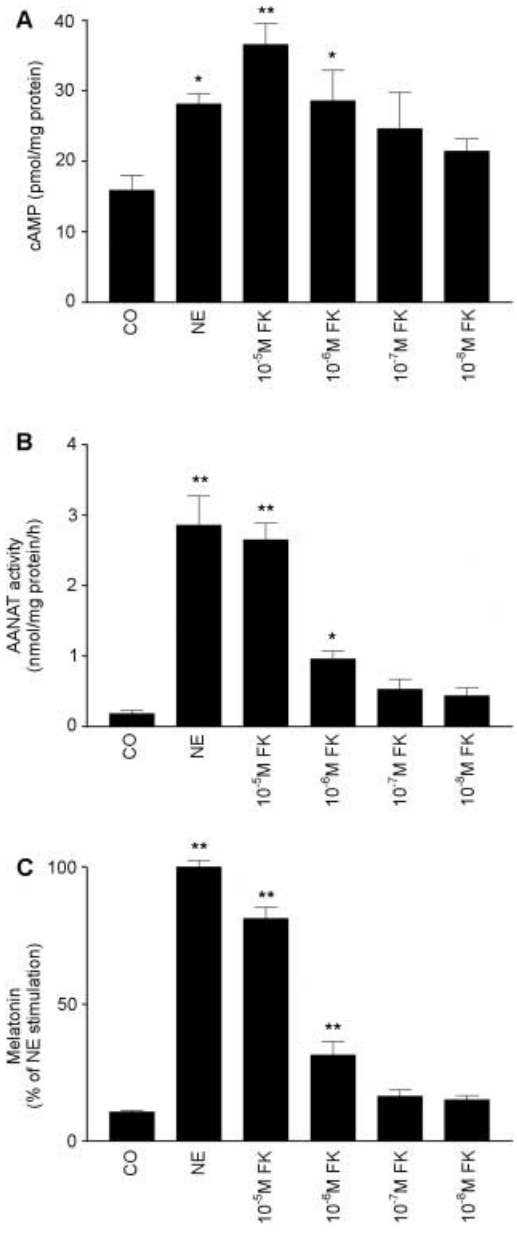

Fig. 3 Effects of norepinephrine (*NE*) and forskolin (*FK*) on intracellular cAMP levels (**A**), AANAT activity (**B**), and melatonin production (**C**). Cells were untreated (*CO*) or stimulated with 10^{-7} M NE or various concentrations of FK without addition of IBMX. After 15 min, samples were prepared for cAMP analysis. AANAT activity and melatonin levels in the culture medium were measured after 6 h. Data were determined in duplicate in each experiment; for data presentation, results from three independent experiments were pooled. For further details, see Materials and methods. **P*<0.05, ***P*<0.001 vs untreated cells

The importance of cAMP in the effects of NE and forskolin were examined using Rp-8-CPT-cAMPS, a selective blocker of cAMP stimulation of protein kinase A. This compound blocked both the NE- and the forskolin-induced increases in AANAT activity in a dose-dependent manner (Fig. 6A).

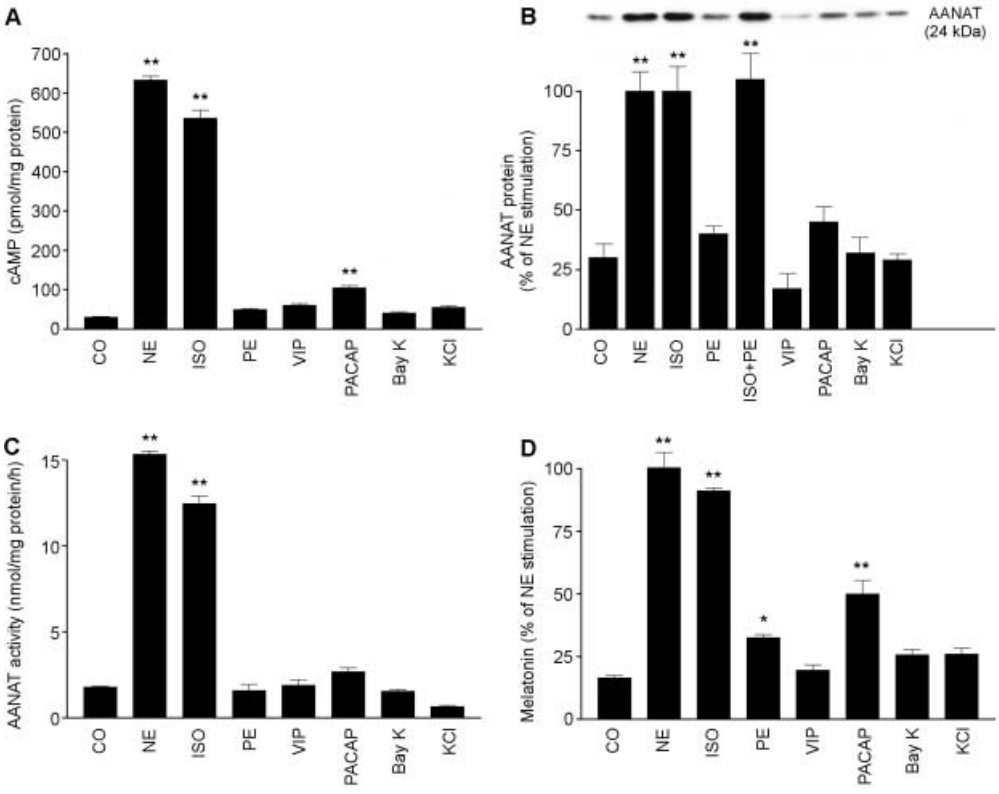

Fig. 4 Effect of adrenergic receptor agonists, VIP, PACAP, and $[Ca^{2+}]_i$-elevating drugs on cAMP accumulation (**A**), AANAT protein levels (**B**), AANAT activity (**C**), and melatonin secretion (**D**). Cells were untreated (*CO*) or stimulated with the indicated drugs. **A** Total accumulated cAMP (i. e. intracellular cAMP plus cAMP secreted into the medium) was measured in cell preparations stimulated with the indicated drugs for 15 min in the presence of IBMX (5×10^{-4} M). **B** AANAT protein levels were calculated semiquantitatively as described (Wicht et al. 1999) and expressed as percentage of the signal obtained after stimulation with NE. **C** AANAT activity was determined in cell homogenates. **D** Melatonin secretion was expressed as percentage of the value obtained after stimulation with *NE*. Data were determined in duplicate in each experiment; for data presentation, results from three independent experiments were pooled. For further details, see Materials and methods. *NE* (norepinephrine; 10^{-7} M), *ISO* (isoproterenol; 10^{-7} M), *PE* (phenylephrine; 10^{-7} M), *ISO+PE* (combined treatment with 10^{-7} M ISO and 10^{-6} M PE), *VIP* (10^{-7} M), *PACAP* (10^{-7} M), *Bay K* 8643 (10^{-6} M), *KCl* (6×10^{-2} M). **P*<0.05, ***P*<0.001 vs untreated cells; NE-treated vs ISO-treated cells: *not significant* (**A**, **B**, **D**) and **P*<0.05 (**C**), respectively

To investigate whether AANAT protein or AANAT activity are regulated through a mechanism that involves α_1/β-adrenergic potentiation, as is the case in the rat, cells were stimulated with various concentrations of ISO (ranging from 10^{-6} M to 10^{-11} M) plus 10^{-6} M PE. No evidence of potentiation was obtained from analysis of AANAT protein levels: in no case did PE markedly increase the response to ISO; the responses to combined treatment with PE and ISO were very similar to that induced by equimolar amounts of ISO alone. A representative experiment with an ISO concentration of 10^{-7} M is shown in Fig. 4B. In contrast, PE clearly potentiated the effect of ISO on AANAT activity over a broad range of concentrations (Fig. 5B).

Of the other agents studied, PACAP (10^{-7} M) treatment resulted in weak increases in AANAT protein levels (Fig. 4B) and AANAT activity (Fig. 4C). Immunocytochemical analyses revealed that PACAP induced changes in AANAT protein levels in a much smaller percentage (~15%) of pinealocytes than did NE (Fig. 8). Treatment with VIP (10^{-7} M), Bay K 8643 (10^{-6} M), or KCl (6×10^{-2} M) did not affect AANAT protein levels or activity (Fig. 4B, C). Likewise, ACh (10^{-4} M), carbachol (10^{-4} M), or glutamate (10^{-3} M) did not change AANAT protein levels (data not shown) or activity (Fig. 7A).

Melatonin

NE (10^{-7} M) treatment for 6 h induced a 5- to 12-fold increase in melatonin accumulation compared with untreated cells (Figs. 3C, 4D, 6B and 7B). The effect of NE was dose- and time-dependent; the lowest effective NE concentration was 10^{-9} M, and maximal responses were obtained at 10^{-7} M NE. Time-course studies indicated that melatonin levels started to increase already within 1 h after NE application, and melatonin gradually accumulated in the course of the experiments (Fig. 9A). Like NE, ISO induced a marked increase in melatonin secretion (Fig. 4D). PE induced a weak increase in melatonin secretion only at high concentrations (10^{-6} M; Fig. 4D) and did not amplify the response induced by various concentrations of ISO (Fig. 5C). The NE-induced melatonin response was mimicked by treatment with 10^{-5} M

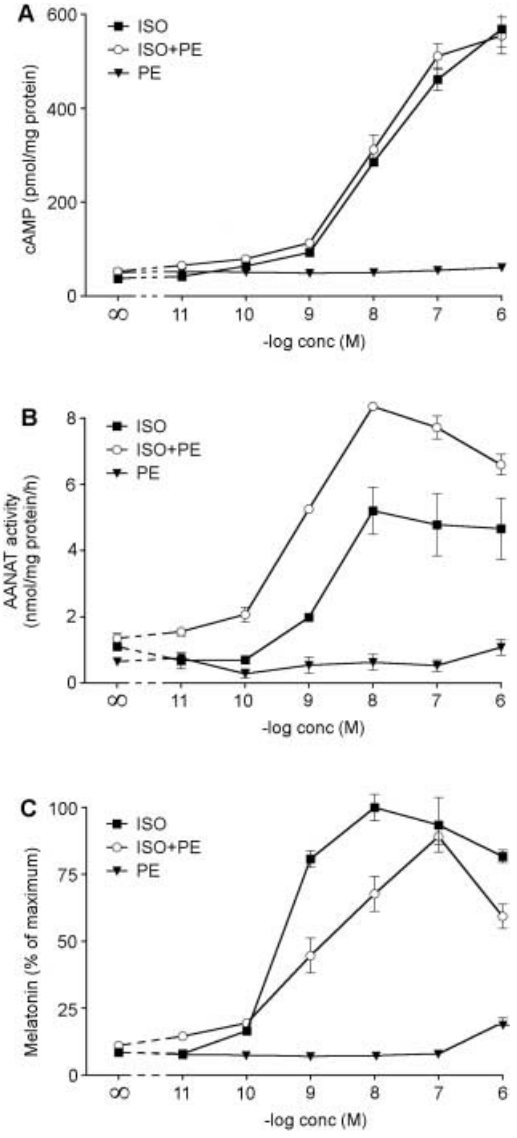

Fig. 5 Dose-response curves for total cAMP accumulation (**A**), AANAT activity (**B**) and melatonin secretion (**C**) induced by phenylephrine (*PE*), isoproterenol (*ISO*), or *ISO* + 10^{-6} M *PE*. Total accumulated cAMP, i.e. the total amount of cAMP extracted from the cells plus cAMP secreted into the supernatants, was determined 15 min after the onset of stimulation in the presence of IBMX. Cells were stimulated with the indicated concentrations of ISO or PE or combinations of a range of concentrations of ISO plus 10^{-6} M PE. Data were determined in duplicate in each experiment; for data presentation, results from two independent experiments were pooled. For further details, see Materials and methods

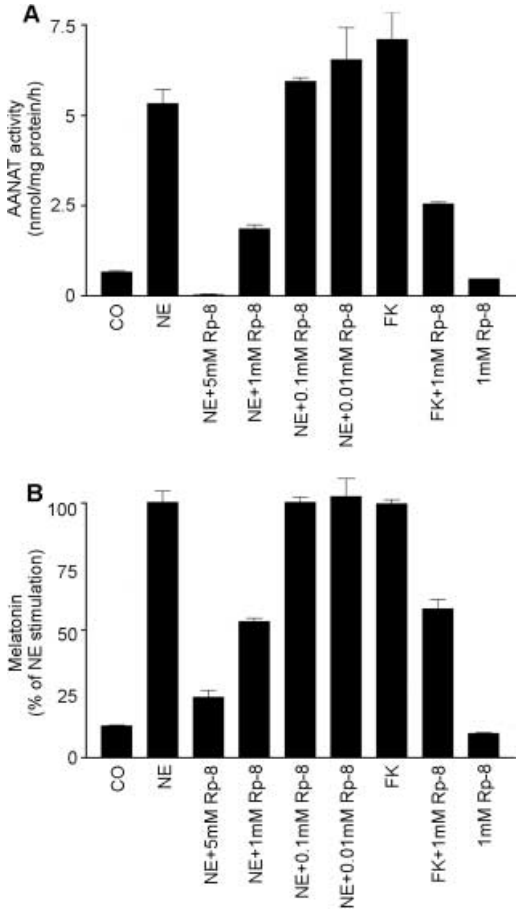

Fig. 6 Effects of the cAMP antagonist Rp-8-CPT-cAMPS (*Rp-8*) on norepinephrine (*NE*)- and forskolin (*FK*)-induced increases in AANAT activity (**A**) and melatonin production (**B**). Cells were pretreated with the indicated concentrations of Rp-8-CPT-cAMPS for 1 h and then stimulated with NE (10^{-7} M) or FK (10^{-5} M) in the presence of the antagonist for 6 h. Note that treatment of cells with Rp-8-CPT-cAMPS (10^{-3} M) alone did not significantly affect AANAT activity or melatonin production compared with unstimulated cells. Data were determined in duplicate in each experiment; for data presentation, results from two independent experiments were pooled. For further details, see Materials and methods

forskolin, but not at lower concentrations (10^{-6} M; 10^{-7} M) (Fig. 3C). The increases in melatonin production induced by NE or forskolin were blocked by Rp-8-CPT-cAMPS in a dose-dependent manner (Fig. 6B).

PACAP at concentrations of ≥10^{-9} M induced a moderate increase in melatonin secretion. The maximal effect was observed at 10^{-7} M PACAP, and this concentration was used in all experiments throughout this study. After treatment with PACAP (10^{-7} M) for 6 h, the melatonin content in the supernatants was approximately 50% of that of NE-treated cells (Fig. 4D). An increase in melatonin was already detectable after 1 h (Fig. 9B). Treatment with VIP (10^{-7} M), Bay K 8643 (10^{-6} M), or KCl (6×10^{-2} M) was without effect (Fig. 4D). Likewise, application of ACh, carbachol (10^{-4} M each) or glutamate (10^{-3} M) for 6 h failed to affect melatonin release (Fig. 7B).

Effects of cholinergic and glutamatergic stimuli on NE-dependent AANAT activity and melatonin secretion

To examine putative interactions of cholinergic, glutamatergic, and adrenergic stimuli on melatonin release, one group of cells was treated for 6 h with a combination of 10^{-7} M NE plus either 10^{-4} M acetylcholine, 10^{-4} M carbachol, or 10^{-3} M glutamate. Co-application of NE plus acetylcholine, carbachol, or glutamate did not significant-

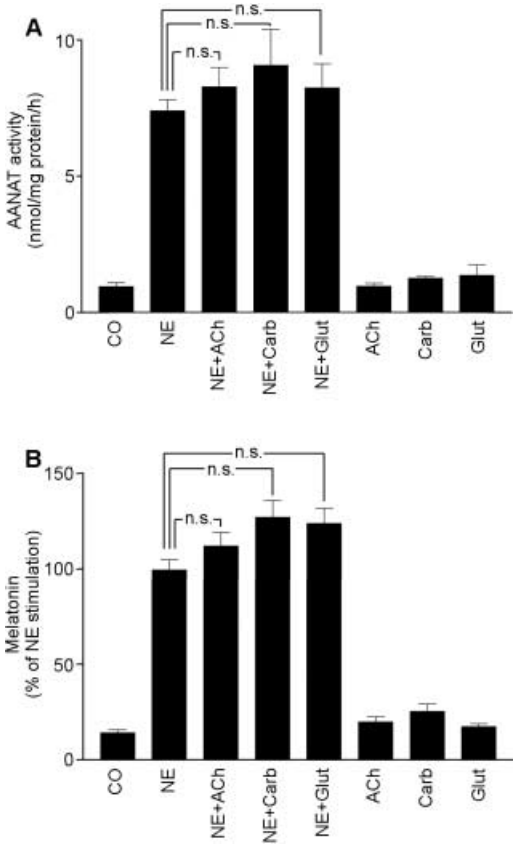

Fig. 7 Effect of acetylcholine receptor agonists and glutamate on basal and norepinephrine (NE)-dependent AANAT enzyme activity (**A**) and melatonin secretion (**B**). Cells were untreated (*CO*) or treated for 6 h with *NE* (10^{-7} M), acetylcholine (*ACh*; 10^{-4} M) carbachol (*Carb*; 10^{-4} M), or glutamate (*Glut*; 10^{-3} M) alone or with combinations of *NE + ACh* or *Carb* or *Glut*. Data were determined in duplicate in each experiment; for data presentation, results from two independent experiments were pooled. For further details, see Materials and methods; *n.s.* not significant

ly affect AANAT activity or melatonin production compared with the effects elicited by NE alone (Fig. 7A, B). A second group was treated with 10^{-7} M NE alone for 0.5 h and then for 5.5 h with a combination of 10^{-7} M NE plus either 10^{-4} M acetylcholine, 10^{-4} M carbachol, or 10^{-3} M glutamate. A third group was treated for 0.5 h with either 10^{-4} M acetylcholine, 10^{-4} M carbachol, or 10^{-3} M glutamate and then for 6 h with a combination of 10^{-7} M NE plus acetylcholine, carbachol, or glutamate. These combined treatments did not significantly alter melatonin secretion compared with the response to treatment with NE (10^{-7} M) alone (data not shown).

Discussion

In the present study, we have used a primary cell culture of bovine pinealocytes to investigate signal transduction cascades regulating the biosynthesis of melatonin in an ungulate species. A comparison between our data obtained with bovine pinealocytes and previous results from rat pinealocytes reveals similarities and differences

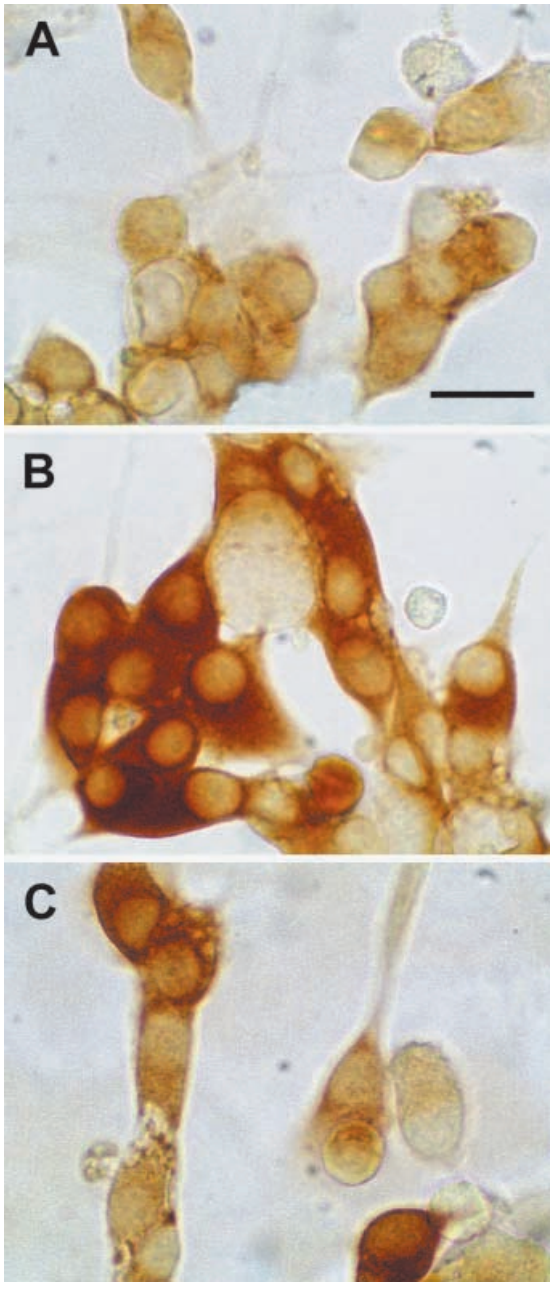

Fig. 8A–C Immunocytochemical detection of arylalkylamine *N*-acetyltransferase (AANAT) in bovine pinealocytes. Cells were cultured for 5 days and treated with the indicated drugs for 6 h. **A** Unstimulated cell preparations were weakly AANAT immunoreactive. **B** Norepinephrine (10^{-7} M) induced a strong AANAT immunoreaction in most pinealocytes. The immunoreaction was confined to granules evenly distributed in the cytoplasm. The nucleus was immunonegative. **C** PACAP (10^{-7} M) induced AANAT immunoreactivity in selected cells only. Note that some cells are weakly AANAT immunoreactive whereas only few cells are strongly AANAT immunoreactive. Similar results were obtained in three independent experiments. For further details, see Materials and methods. *Scale bar* 15 µm for all panels

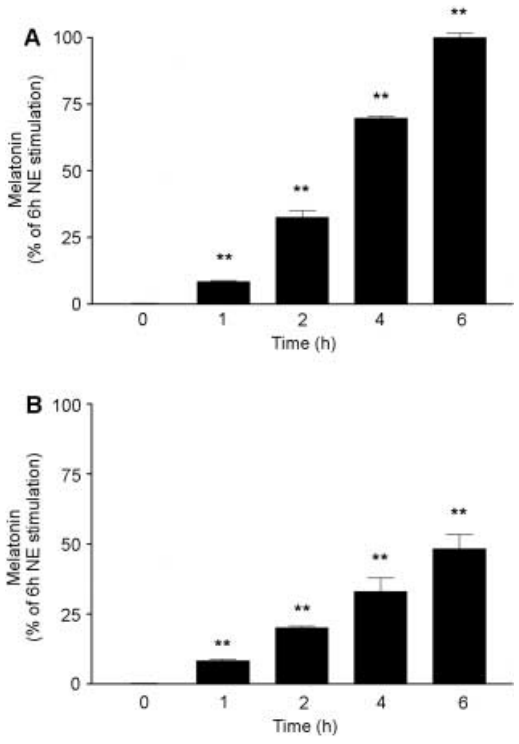

Fig. 9A, B Time-courses of norepinephrine (NE)- and PACAP-induced melatonin secretion. Bovine pinealocytes were stimulated with 10^{-7} M NE (**A**) or 10^{-7} M PACAP (**B**) for the indicated time periods. For each time point, melatonin was determined in the supernatants of untreated and stimulated cells, and the basal melatonin production in untreated cells was substracted from the corresponding values obtained after treatment with NE or PACAP, respectively. The results are expressed as percent of the value recorded after 6 h stimulation with NE. Data were determined in duplicate in each experiment; for data presentation, results from two independent experiments were pooled. For further details, see Materials and methods. $^{**}P<0.001$ vs untreated cells

in these cascades between these two mammalian species (Table 1; summarizing data of the present study and previous investigations). This supports the concept that a well-conserved neuroendocrine effector mechanism, i.e. the production and release of melatonin at night, can be controlled through multiple molecular mechanisms (cf. Klein et al. 1997; Korf 1998; Korf et al. 1998) and emphasizes the need for comparative studies to understand the regulation of pineal melatonin biosynthesis in the pineal organ of each species of interest.

The results of the present investigation indicate that a notable feature of regulation of melatonin production in the rat pinealocyte is also evident in the bovine pinealocyte, i.e. that NE is the most potent of several transmitter substances. NE activates both α_1- and β-adrenergic receptors. In both species, selective stimulation of β-adrenergic receptors leads to a marked accumulation of cAMP, but leaves $[Ca^{2+}]_i$ unchanged; and, α_1-selective ligands induce a strong increase in $[Ca^{2+}]_i$ in most cells, but do not elevate cAMP levels.

These results emphasize the dominant role of β-adrenergic control of melatonin synthesis in the bovine pineal gland and are in line with previous studies (Chan and Ebadi 1980; Rüppel and Olcese 1991). Furthermore, our data show that the NE-induced rise in intracellular cAMP is essential for melatonin synthesis and that inhibition of cAMP-directed processes by blockade of protein kinase A largely abolishes the increase in melatonin production. Thus, it is evident that, in both these mammals, the dominant mechanism controlling melatonin production involves adrenergic regulation of cAMP, although the NE-induced increases in cAMP levels are less prominent in the bovine (twofold) than in the rat (100-fold; Vanecek et al. 1985). The basis for this difference is not clear.

One interesting implication of our studies is that the increase in cAMP levels alone is not sufficient to fully activate the melatonin biosynthesis in bovine pinealocytes. This is evident from the comparison of the effects of NE and forskolin on cAMP levels, AANAT activity, and melatonin production. Both 10^{-7} M NE and low doses of forskolin increase cAMP levels twofold, but these treatments do not affect AANAT activity and melatonin synthesis in a similar manner: forskolin is distinctly less potent. This finding suggests that additional NE-driven mechanisms further enhance AANAT activity and melatonin production, which potentiate the effects of cAMP, but do not act via the cAMP-pathway. One mechanism may involve $[Ca^{2+}]_i$, which is markedly elevated by NE via activation of α_1-adrenergic receptors, but not by forskolin. By itself, α_1-adrenergic stimulation of bovine pinealocytes does not stimulate any of the downstream parameters investigated, but it does potentiate the β-adrenergically induced increase in AANAT activity. With regard to the molecular basis of α_1/β-adrenergic interaction, it is important to point out that potentiation of AANAT activity in the bovine pinealocyte is not accompanied by potentiating effects on cAMP levels. This suggests that the α_1-adrenergic potentiation of AANAT activity in the bovine pinealocyte occurs downstream to the formation of cAMP. Similar downstream effects have been described in rat pinealocytes (Yu et al. 1993), but in this species $[Ca^{2+}]_i$ has a second site of regulation of AANAT activity: it potentiates the increases in cAMP levels (Vanecek et al. 1985) via activation of protein kinase C (Sugden et al. 1985). Another mechanism may involve cGMP, which has been shown to be elevated by NE in bovine pinealocytes (Maronde et al. 1995). Although forskolin also elevates cGMP in rat pinealocytes (Ho et al. 1989), it is not known whether this occurs in the bovine pinealocyte.

In addition, α_1-adrenergic potentiation of β-adrenergically induced AANAT activity in the bovine pinealocyte is also not accompanied by a potentiating effect on melatonin production. The molecular basis for this finding is unclear.

The temporal patterns of melatonin secretion after adrenergic stimulation also differ between bovine and rat. In the rat pineal organ, there is a 2-h lag period before melatonin production increases significantly after onset of NE treatment (Klein 1985; Pfeffer et al. 1999), re-

Table 1 Comparative synopsis of bovine and rat pineal signal transduction cascades. The effects of adrenergic, peptidergic, cholinergic, glutamatergic stimuli and of voltage-gated calcium channel activation, on key components/parameters of the melatonin-generating system as determined in the present investigation and in previous studies are summarized. The intensities of the effects are classified as follows: – no effect, + weak effect, ++ moderate effect, +++ strong effect, *n.d.* not determined (*NE* norepinephrine, *VGCC* voltage-gated calcium channels, *nic* nicotinergic, *musc* muscarinergic)

Stimulation	$[Ca^{2+}]_i$		Cyclic AMP		AANAT mRNA		AANAT activity/protein		Melatonin	
	Bovine	Rat	Bovine	Rat	Bovine	Rat	Bovine	Rat	Bovine	Rat
β-Adrenergic	–[1]	–[2, 3, 4]	+[1, 5]	+++[6]	–[7]	+++[8]	+++[1, 7]	+++[9]	+++[1, 5]	+++[6, 9]
α_1-Adrenergic										
alone	++[1]	+++[2, 3, 4]	–[1, 5]	–[6]	n.d.	–[8]	–[1, 7, 10]	–[9]	–[1, 5]	–[6, 9]
with β-adrenergic			–[1, 5]	potentiation[11]	n.d.	n.d.	potentiation[1]	potentiation[12, 13]	–[1, 5]	++[12]
VIP	–[1]	–[14, 15]/+[16]	–[1]	+[17]	n.d.	+[8]	–[1]	+[17, 18]	–[1]	+[19]
PACAP	–[1]	–[14]/+[15, 20]	+[1]	+[21]	n.d.	n.d.	+[1]	+[22]	++[1]	++[23]
VGCC activators	++[1]	+++[2, 24]	–[1]	–[25]	n.d.	n.d.	–[1]	n.d.	–[1]	–[26]
Acetylcholine										
alone	++[1] (nic, musc)	+++[4, 24] (nic)	–[1]	n.d.	n.d.	n.d.	–[1]	–[27, 28]	–[1]	–[27, 29]
with NE			–[1]	inhibitory[27]	n.d.	n.d.	–[1]	inhibitory[27]	–[1]	inhibitory[27, 29]
Glutamate										
alone	–[1]	+[30]	–[1]	–[30]	n.d.	n.d.	–[1]	–[30]	–[1]	–[30, 31]
with NE			–[1]	inhibitory[30]	n.d.	n.d.	–[1]	inhibitory[30]	–[1]	inhibitory[30, 31]

The data are derived from the following sources: [1]present investigation; [2]Sugden et al. (1987); [3]Schaad et al. (1993); [4]Schomerus et al. (1995); [5]Rüppel and Olcese (1991); [6]Klein et al. (1997); [7]Schomerus et al. (2000); [8]Roseboom et al. (1996); [9]Klein (1985); [10]Chan and Ebadi (1980); [11]Vanecek et al. (1985); [12]Klein et al. (1983); [13]Yu et al. (1993); [14]Schomerus et al. (1996); [15]Olcese et al. (1996); [16]Schaad et al. (1995); [17]Kaneko et al. (1980); [18]Yuwiler (1983); [19]Simonneaux et al. (1990); [20]Darvish and Russell (1998); [21]Chik and Ho (1995); [22]Yuwiler et al. (1995); [23]Simonneaux et al. (1993); [24]Letz et al. (1997); [25]Sugden et al. (1986); [26]Pfeffer et al. (1999); [27]Yamada et al. (1998a); [28]Buda and Klein (1978); [29]Stankov et al. (1993); [30] Yamada et al. (1998b); [31] Kus et al. (1994)

flecting the time required for AANAT mRNA to accumulate (Coon et al. 1995; Roseboom et al. 1996). In contrast, melatonin production in bovine pinealocytes was markedly enhanced already within 1 h after the onset of NE treatment. This rapid response is consistent with the finding that AANAT mRNA in ungulates is expressed at relatively high levels in the unstimulated gland (Coon et al. 1995; Klein et al. 1996); and, that NE rapidly increases AANAT protein levels and activity by rapidly controlling proteasomal proteolysis of the enzyme, without requiring changes in expression of the AANAT gene (Schomerus et al. 2000). The rapid increase in melatonin production seen with the bovine pinealocyte in vitro is also seen in vivo: blood and cerebrospinal fluid melatonin levels increase rapidly after the onset of darkness (Hedlund et al. 1977).

In vitro studies indicate that, in addition to NE, VIP and PACAP influence pineal functions in the rat. While the effect of VIP and PACAP on $[Ca^{2+}]_i$ in rat pinealocytes is controversial (Schaad et al. 1995; Schomerus et al. 1996; Olcese et al. 1996; Darvish and Russell 1998; see Table 1), it is well established that both neuropeptides increase cAMP levels, AANAT activity (Kaneko et al. 1980; Yuwiler 1983; Chik and Ho 1995), and melatonin synthesis (Simonneaux et al. 1990, 1993; Pfeffer et al. 1999), albeit to lesser degree than NE. As seen with NE, the VIP- and PACAP-induced increases in melatonin synthesis in the rat were delayed for 2–4 h after the onset of the treatment (Pfeffer et al. 1999), suggesting that both neuropeptides also act via transcriptional regulation of AANAT. As shown in the present study, PACAP weakly elevates cAMP, AANAT activity, and AANAT protein and leads to a moderate increase in melatonin biosynthesis in the bovine pinealocyte. These effects were smaller than those due to NE treatment, apparently because the effects of PACAP are restricted to a subpopulation of bovine pinealocytes, as shown by immunocytochemical data presented in this report. As observed with NE, the effects of PACAP on melatonin synthesis were rapid, consistent with the interpretation that PACAP – like NE – acts primarily via posttranscriptional mechanisms in the bovine pinealocyte. Thus, the PACAP signal seems to be processed differently in rat and bovine pinealocytes.

In contrast to the rat, we found that VIP does not activate $[Ca^{2+}]_i$, cAMP, AANAT protein, AANAT activity, or melatonin synthesis in the bovine pinealocyte. Our data indicate qualitative differences in neuropeptidergic signaling between the two species.

The role of cholinergic signal transduction cascades in mammalian pineal function, especially in the regulation of melatonin production, is not fully understood. In

neonatal rat pinealocytes, ACh increases $[Ca^{2+}]_i$ exclusively via muscarinic ACh receptors (Schomerus et al. 1999). In the first two postnatal weeks, the muscarinic signaling is gradually shifted to nicotinic signaling and, in adult rat pinealocytes, ACh was demonstrated to elevate $[Ca^{2+}]_i$ exclusively via nicotinic ACh receptors; activation of muscarinic receptors did not contribute to this response any longer (Schomerus et al. 1995; Letz et al. 1997). Activation of nicotinic ACh receptors triggers L-glutamate exocytosis from pineal microvesicles. Reportedly, L-glutamate inhibits NE-induced melatonin synthesis through paracrine and/or autocrine mechanisms (Yamada et al. 1998a, 1998b). Like in the adult rat, selective stimulation of nicotinic ACh receptors was also found to induce an increase in $[Ca^{2+}]_i$ in bovine pinealocytes, apparently via influx of calcium ions into the cells through nifedipine-sensitive voltage-gated calcium channels. Surprisingly, the majority of bovine pinealocytes also responded to stimulation of muscarinic ACh receptors. The present demonstration of functional ACh receptors in bovine pinealocytes conforms to immunohistochemical investigations revealing a cholinergic innervation (Phansuwan-Pujito et al. 1991) and biochemical investigations showing binding sites for cholinergic ligands (Govitrapong et al. 1989) in the bovine pineal gland. In contrast to rat pinealocytes, however, cholinergic and glutamatergic stimuli do not inhibit NE-induced increases in AANAT activity or in melatonin production in bovine pinealocytes. Thus, rat and bovine pinealocytes also differ with regard to cholinergic signalling. The functional significance of ACh receptors and also that of voltage-gated calcium channels in bovine pinealocytes remains to be elucidated.

In conclusion, many aspects of melatonin production in the bovine pinealocyte appear similar to those in primates; in both cases, melatonin production seems to increase rapidly following exposure to darkness at night, suggesting posttranscriptional control mechanisms. This points to the bovine pinealocyte as a practical and potentially useful model to elucidate the molecular basis of regulation of melatonin production in non-rodent species, including primates.

References

Arendt J (1995) Melatonin and the mammalian pineal gland. Chapman and Hall, London

Baler R, Covington S, Klein DC (1997) The rat arylalkylamine *N*-acetyltransferase gene promoter. J Biol Chem 272:6979–6985

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Buda M, Klein DC (1978) A suspension culture of pinealocytes: regulation of *N*-acetyltransferase activity. Endocrinology 103: 1483–1493

Chan A, Ebadi M (1980) The kinetics of norepinephrine-induced stimulation of serotonin *N*-acetyltransferase in bovine pineal gland. Neuroendocrinology 31:244–251

Chik CL, Ho AK (1995) Pituitary adenylate cyclase-activating polypeptide: control of rat pineal cyclic AMP and melatonin but not cyclic GMP. J Neurochem 64:2111–2117

Coon SL, Roseboom PH, Baler R, Weller JL, Namboodiri MAA, Koonin EV, Klein DC (1995) Pineal serotonin *N*-acetyltransferase: expression cloning and molecular analysis. Science 270:1681–1683

Darvish N, Russell JT (1998) Neurotransmitter-induced novel modulation of a nonselective cation channel by a cAMP-dependent mechanism in rat pineal cells. J Neurophysiol 79:2546–2556

Drijfhout WA, van der Linde S, Kooi C, Grol B, Westerink BHC (1996) Norepinephrine release in the rat pineal gland: the input from the biological clock measured by in vivo microdialysis. J Neurochem 66:748–755

Falcón J, Galarneau KM, Weller JL, Ron B, Chen G, Coon SL, Klein DC (2001) Regulation of arylalkylamine *N*-acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the fish pineal organ: evidence for a role of proteasomal proteolysis. Endocrinology 142:1804–1813

Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, Namboodiri MAA, Coon SL, Hickman AB, Rollag M, Obsil T, Beauverger P, Ferry G, Boutin JA, Klein DC (2001) Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14–3-3 binding switch in melatonin synthesis. Proc Natl Acad Sci USA 98:8083–8088

Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC (1998) Melatonin production: proteosomal proteolysis in serotonin *N*-acetyltransferase regulation. Science 279:1358–1360

Govitrapong P, Phansuwan-Pujito P, Ebadi M (1989) Studies on the properties of muscarinic cholinergic receptor sites in bovine pineal gland. Comp Biochem Physiol C Toxicol Pharmacol 94:159–164

Hedlund L, Lischko MM, Rollag MD, Niswender GD (1977) Melatonin: daily cycle in plasma and cerebrospinal fluid of calves. Science 195:686–687

Ho AK, Chik CL, Klein DC (1989) Forskolin stimulates pinealocyte cGMP accumulation: dramatic potentiation by an α_1-adrenergic $\rightarrow [Ca^{2+}]_i$ mechanism involving protein kinase C. FEBS Lett 249:207–212

Iuvone PM, Brown AD, Haque R, Weller JL, Zawilska JB, Chaurasia SS, Ma MH, Klein DC (2002) Retinal melatonin production: role of proteasomal proteolysis in circadian and photic control of arylalkylamine *N*-acetyltransferase. Invest Ophthalmol Vis Sci 43:564–72

Kaneko T, Cheng PY, Oka H, Oda T, Yanaihara N, Yanaihara C (1980) Vasoactive intestinal polypeptide stimulates adenylate cyclase and serotonin *N*-acetyltransferase activities in rat pineal gland in vitro. Biomed Res 1:84–87

Khalil EM, Cole PA (1998) A potent inhibitor of the melatonin rhythm enzyme. J Am Chem Soc 120:6195–6196

Klein DC (1985) Photoneural regulation of the mammalian pineal gland. In: Evered D, Clark S (eds) Photoperiodism, melatonin and the pineal gland. Ciba Foundation Symposium 117. Pitman, London, pp 38–56

Klein DC, Weller JL (1970) Indole metabolism in the pineal gland: a circadian rhythm in N-acetyltransferase. Science 169: 1093–1095

Klein DC, Sugden D, Weller JL (1983) Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal serotonin *N*-acetyltransferase. Proc Natl Acad Sci USA 80: 599–603

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Bégay V, Falcón J, Cahill GM, Cassone VM, Baler, R (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. Rec Progr Hormone Res 52:307–358

Korf HW (1998) Evolution and regulation of neurosecretory cells. In: Yagihashi S, Kachi T, Wakui M (eds) Dynamic cells: cell biology of the 21st century. Elsevier, Amsterdam, pp 31–45

Korf HW, Schomerus C, Maronde E, Stehle JH (1996) Signal transduction molecules in the rat pineal organ: Ca^{2+}, pCREB, and ICER. Naturwissenschaften 83:535–543

Korf HW, Schomerus C, Stehle JH (1998) The pineal organ, its hormone melatonin, and the photoneuroendocrine system. Adv Anat Embryol Cell Biol 146: 1–100

Kus L, Handa RJ, McNulty JA (1994) Glutamate inhibition of the adrenergic-stimulated production of melatonin in rat pineal gland in vitro. J Neurochem 62:2241–2245

Letz B, Schomerus C, Maronde E, Korf HW, Korbmacher C (1997) Stimulation of a nicotinic ACh receptor causes depolarization and activation of L-type Ca^{2+} channels in rat pinealocytes. J Physiol 499:329–340

Maronde E, Middendorff R, Mayer B, Olcese J (1995) The effect of NO-donors in bovine and rat pineal cells: stimulation of cGMP and cGMP-independent inhibition of melatonin synthesis. J Neuroendocrinol 7:207–214

Maronde E, Pfeffer M, Olcese J, Molina CA, Schlotter F, Dehghani F, Korf HW, Stehle JH (1999) Transcription factors in neuroendocrine regulation: rhythmic changes in pCREB and ICER levels frame melatonin synthesis. J Neurosci 19: 3226–3336

Obsil T, Ghirlando R, Klein DC, Ganguly S, Dyda F (2001) Crystal structure of the 14-3-3 zeta/serotonin *N*-acetyltransferase complex: a role for scaffolding in enzyme regulation. Cell 105: 257–267

Olcese J, McArdle C, Mikkelsen J, Hannibal J (1996) PACAP and type I PACAP receptors in the pineal gland. Ann NY Acad Sci 805:595–600

Pfeffer M, Maronde E, Molina CA, Korf HW, Stehle JH (1999) Inducible cyclic AMP early repressor protein in rat pinealocytes: a highly sensitive natural reporter for regulated gene transcription. Mol Pharmacol 56:279–289

Phansuwan-Pujito P, Mikkelsen JD, Govitrapong P, Møller MA (1991) Cholinergic innervation of the bovine pineal gland visualized by immunohistochemical detection of choline acetyltransferase-immunoreactive nerve fibers. Brain Res 545:49–58

Roseboom PH, Klein DC (1995) Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: involvement of a β-adrenergic/cyclic AMP mechanism. Mol Pharmacol 47:439–449

Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC (1996) Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. Endocrinology 137: 3033–3044

Rüppel R, Olcese J (1991) Bovine pinealocytes in monolayer culture: studies on the adrenergic regulation of melatonin secretion. Endocrinology 129:2655–2662

Schaad NC, Parfitt A, Russell JT, Schaffner AE, Korf HW, Klein DC (1993) Single-cell $[Ca^{2+}]_i$ analysis and biochemical characterization of pinealocytes immobilized with novel attachment peptide preparation. Brain Res 614:251–256

Schaad NC, Vanecek J, Rodriguez IR, Klein DC, Holtzclaw L, Russell JT (1995) Vasoactive intestinal peptide elevates pinealocyte intracellular calcium concentrations by enhancing influx: evidence for involvement of a cyclic GMP-dependent mechanism. Mol Pharmacol 47:923–933

Schomerus C, Laedtke E, Korf HW (1995) Calcium responses of isolated, immunocytochemically identified rat pinealocytes to noradrenergic, cholinergic and vasopressinergic stimulations. Neurochem Int 27:163–175

Schomerus C, Maronde E, Laedtke E, Korf HW (1996) Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) induce phosphorylation of the transcriptional factor CREB in subpopulations of rat pinealocytes: immunocytochemical and immunochemical evidence. Cell Tissue Res 286:305–313

Schomerus C, Laedtke E, Korf HW (1999) Analyses of signal transduction cascades in rat pinealocytes reveal a switch in cholinergic signaling during postnatal development. Brain Res 833:39–50

Schomerus C, Korf HW, Laedtke E, Weller JL, Klein DC (2000) Selective adrenergic/cyclic AMP-dependent switch-off of proteasomal proteolysis alone switches on neural signal transduction: an example from the pineal gland. J Neurochem 75: 2123–2132

Simonneaux V, Ouichou A, Pévet P, Masson-Pévet M, Vivien-Roels B, Vaudry H (1989) Kinetic study of melatonin release from rat pineal glands using a perifusion technique. J Pineal Res 7: 63–83

Simonneaux V, Ouichou A, Pévet P (1990) Vasoactive intestinal peptide stimulates melatonin release from perifused pineal glands of rats. J Neural Transm 79:69–79

Simonneaux V, Ouichou A, Pévet P (1993) Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates melatonin synthesis from the rat pineal gland. Brain Res 603:148–152

Stankov B, Cimino M, Marini P, Lucini V, Fraschini F, Clementi F (1993) Identification and functional significance of nicotinic cholinergic receptors in the rat pineal gland. Neurosci Lett 156:131–134

Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB (1985) Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314:359–360

Sugden AL, Sugden D, Klein DC (1986) Essential role of calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. J Biol Chem 261:11608-11612

Sugden LA, Sugden D, Klein DC (1987) α_1-Adrenergic activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. J Biol Chem 262:741–745

Tamotsu S, Schomerus C, Stehle JH, Roseboom PH, Korf HW (1995) Norepinephrine-induced phosphorylation of the transcription factor CREB in isolated rat pinealocytes: an immunocytochemical study. Cell Tissue Res 282:219–226

Vanecek J, Sugden D, Weller JL, Klein DC (1985) Atypical synergistic α_1- and β-adrenergic regulation of adenosine 3', 5'-monophosphate and guanosine 3', 5'-monophosphate in rat pinealocytes. Endocrinology 116:2167–2173

Wicht H, Maronde E, Olcese J, Korf HW (1999) A semiquantitative image-analytical method for the recording of dose-response curves in immunocytochemical preparations. J Histochem Cytochem 47:411–417

Yamada H, Ogura A, Koizumi S, Yamaguchi A, Moriyama Y (1998a) Acetylcholine triggers L-glutamate exocytosis via nicotinic receptors and inhibits melatonin synthesis in rat pinealocytes. J Neurosci 18:4946–4952

Yamada H, Yastushiro S, Ishio S, Hayashi M, Nishi T, Yamamoto A, Futai M, Yamaguchi A, Moriyama Y (1998b) Metabotropic glutamate receptors negatively regulate melatonin synthesis in rat pinealocytes. J Neurosci 18:2056–2062

Yu L, Schaad NC, Klein DC (1993) Calcium potentiates cyclic AMP stimulation of pineal arylalkylamine *N*-acetyltransferase. J Neurochem 60:1436–43

Yuwiler A (1983) Vasoactive intestinal peptide stimulation of pineal serotonin-*N*-acetyltransferase activity: general characteristics. J Neurochem 41:146–153

Yuwiler A, Brammer GL, Bennett BL (1995) Interaction between adrenergic and peptide stimulation in the rat pineal: pituitary adenylate cyclase-activating peptide. J Neurochem 64:2273–2280